Am J Cancer Res 2022;12(5):2146-2159
www.ajcr.us /ISSN:2156-6976/ajcr0142676

Original Article

Gastric cancer with enhanced apical junction pathway has increased metastatic potential and worse clinical outcomes

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Received March 3, 2022; Accepted April 14, 2022; Epub May 15, 2022; Published May 30, 2022

Abstract: Excessive intercellular connection at confluency may be limiting further cell growth or a sign of aggressive biology in the cell culture. As apical junction complex is a main component of cell-to-cell connection, we aimed to investigate gastric cancer biology using Apical Junction Pathway score that we generated using Gene set variant analysis (GSVA) of the "Hallmark Apical Junction" gene set. 1,239 gastric cancer patients from the Cancer Genome Atlas (TCGA) and two GSE cohorts were included in this study. The cohorts were dichotomized using the median of the score. Apical Junction Pathway score high gastric cancer was not consistently associated with increased cell proliferation or immune cell infiltration. On the other hand, Apical Junction Pathway score high gastric cancer was associated with significantly higher infiltration of stromal cells, such as endothelial cells; hence, increased neovascularization and angiogenesis in the tumor microenvironment (TME) were speculated. Gene set enrichment analysis (GSEA) confirmed increased expression of epithelial mesenchymal transition (EMT) and angiogenesis in the high Apical Junction Pathway score group (false discovery rate (FDR) <0.25). Lastly, the high Apical Junction Pathway score group was associated with more aggressive clinicopathological characteristics, such as significantly higher American Joint Committee on Cancer (AJCC) T-category and higher pathological stage, leading to worse disease-specific survival and overall survival (P<0.05, respectively). In conclusion, enhanced Apical Junction Pathway score gastric cancer was associated with aggressive clinical characteristics leading to shorter survival likely due to increased metastatic potential from EMT and angiogenesis.

Keywords: Apical junction, gastric cancer, TCGA, bioinformatics

Introduction

It is well known that when cells reach confluency in the cell culture, excessive intercellular connection compromise cell survival and further growth despite enough nutrition [1]. While confluency in the cell culture dish is commonly seen in the *In Vitro* setting, it is unclear whether similar mechanism occur in *In Vivo* setting since tumor microenvironment (TME) *In Vivo* is much more complex. Intercellular cell-to-cell connection is one of the most important components to maintain cell polarity and the tissues. In epithelial cells, apical junction complex is formed by tight junctions and adherens junctions [2]. A complex set of transmembrane and peripheral proteins constitute both tight junction and adherens junctions, such as E-cadherin, Beta-Catenin, claudins, occludin and more [3, 4]. Cancer cells with excessive intercellular connection may have worse prognosis reflecting aggressive proliferation ability that can achieve confluency, or it may have better prognosis due to plateaued proliferation by reaching confluency.

With significant advance in technology, computational biology using RNA-Sequence (RNA-Seq)

has been utilized more frequently. The single-sample gene set variant analysis (GSVA) is a computational methodology, which can explore the biological activity of a signaling pathway of interest, and has been used to obtain a score from the signaling pathway [5-8]. The benefit of GSVA is that this approach can take coordination of multiple gene sets into account and increase the explanatory power of the model [9, 10].

Gastric cancer is the fifth most diagnosed malignancy worldwide [11] and the third most common cause of cancer-related death globally in 2018 [12, 13]. Treatment for advanced gastric cancer remain challenging despite significant advance in systemic chemotherapy and treatment strategy [11, 14]. The most comprehensive genomic classification in gastric cancer was published by The Cancer Genome Atlas (TCGA) in 2014 [15], yet integration of this genomic classification into the treatment strategy is still in progress. With further advance in molecular analysis, we ought to identify prognostic biomarkers to predict tumor biology as well as therapeutic response in gastric cancer. Given multiple proteins and various genes associated with Apical Junction Pathway, we elected to use GSVA to obtain Apical Junction Pathway score to dichotomize three large gastric cancer cohorts in order to analyze gastric cancer biology. We utilized gastric cancer cohorts for this study, as the molecular pathogenesis of gastric cancer is significantly associated with E-cadherin, which harbors abnormalities in both germline and sporadic gastric cancers [16].

The current study was aimed to investigate if gastric cancer with high Apical Junction Pathway score would 1) have significant proliferation ability leading to worse outcome or 2) halt proliferation by reaching confluency leading to improved outcome.

Materials and methods

Data acquisition from TCGA-stomach adenocarcinoma, GSE84437 and GSE26253 cohorts

Clinicopathological data for the TCGA-stomach adenocarcinoma (STAD) was obtained from the Pan-Cancer Clinical Data Resource [17] and through cBio Cancer Genomics Portal [18], as previously described [19-26]. Transcriptomic data of primary tumor samples with HT-Seq software from Genome Data Commons (GDC) portal of National Cancer Institute (NIH) (https://cancergenome.nih.gov/, USA) using TCGA biolinks [27]. TCGA-STAD cohort includes 440 patients, of which 375 patients were identified to have both gene expressions from RNA-sequence, clinicopathological data and survival data. Furthermore, we identified two more large gastric cancer cohorts (GSE26253 and GSE84437) with transcriptomic data, both of which included 432 patients each [28-30]. While TCGA contained overall survival (OS) and disease specific survival (DSS), both GSE26253 and GSE84437 contained only OS information. With TCGA and two other cohorts being de-identified publicly accessible database, Institutional Review Board (IRB) was waived.

Gene set expression analyses

Log_2-transformed normalized gene expression data was used. The GSVA method [5] was utilized to obtain a GSVA score from gene expression data for the "HALLMARK_APICAL_JUNCTION" gene set of the Molecular Signatures Database Hallmark gene set collection [31]. GSVA Bioconductor package (version 3.10) were used. Within each cohort, tumor samples were categorized into high and low apical junction pathway score groups using the median GSVA score as cut-off. For gene set enrichment analysis (GSEA) [32], GSEA software (version 4.1.0) and the Hallmark gene set collection were used, as we described previously [33-38]. False discovery rate (FDR) threshold of 0.25 was used to deem significance.

Statistical analysis

Statistical analyses were performed using R software (version 4.1.0, http:///www.r-project.org/) and Bioconductor (http://bioconductor.org/). OS was defined as the time from the date of diagnosis to the date of death by any cause and DSS as the time from the date of diagnosis to the date of death by gastric cancer. Kaplan-Meier method with log-rank test was performed for survival analyses. Mann-Whitney U test, Kruskal-Wallis test or Fisher's exact test were used to determine the significance of differ-

ences for groups. A two-sided p value <0.05 was considered statistically significant.

Results

Gastric cancer with activated apical junction pathway was not consistently associated with cell proliferation

Activation of Apical Junction Pathway in gastric cancer was quantified using the GSVA score based on the Hallmark Apical Junction gene set in all three cohorts (Figure S1). Each gastric cancer cohort was dichotomized into the high and low group by the median value of Apical Junction Pathway score.

Since Apical Junction Pathway is activated when cancer cells reached confluency in the cell culture dish, it was of interest whether gastric cancer patients with enhanced Apical Junction Pathway was associated with increased or decreased cell proliferation. While MKI67 expression and proliferation markers were lower in the Apical Junction Pathway enhanced group in the GSE84437 cohort, there was no difference in MKI67 expression or grade by Apical Junction Pathway activation in TCGA (**Figure 1A**). Furthermore, we used GSEA to investigate if the high Apical Junction Pathway score group would enrich any cell proliferation-related gene sets, such as G2M CHECKPOINT, E2F TARGET and MYC TARGETS V1/V2. We found that there was no consistent enrichment of cell proliferation-related gene sets, although there was a trend to enrich to the low score group in both of the cohorts (**Figure 1B**). Therefore, there was a trend that cell proliferation was less in the high Apical Junction Pathway score group, however, without consistent significance in two cohorts.

Gastric cancer with activated apical junction pathway was not associated with uniformly high infiltration of immune cells

Given that Apical Junction Pathway is activated with cell-cell contact, and that abundant presence of tumor infiltrating lymphocytes (TILs) in the tumor microenvironment (TME) is often a favorable marker in survival in multiple cancer types [39, 40], we investigated if gastric cancer with high score was associated with high infiltration of immune cells. Indeed, some of gastric cancers such as Epstein-Barr virus (EBV) positive or microsatellite instability high (MSI-high) gastric cancers have increased mutation burden and enhanced immunity [15]. On the contrary to our expectation, silent and non-silent mutation rate as well as copy number alteration that are known to attract immune cells were all lower in the high Apical Junction Pathway score group (**Figure 2A**). We further examined immune cell composition using xCell algorithm in the TME of both high and low Apical Junction Pathway score groups. Similarly, there were no uniform infiltration of immune cells by the Apical Junction Pathway score; CD4 memory T cell, T Helper type 1 cells and NK cells were low while M1 macrophages were high in the high score group (**Figure 2B**). These results suggest that activated Apical Junction Pathway does not reflect infiltration of immune cells.

Gastric cancer with high apical junction pathway score was associated with high fraction of stromal cells including fibroblasts, adipocytes and vascular endothelial cells in tumor microenvironment

Since Apical Junction Pathway Score was not associated with cell proliferation or with immune cell infiltration that were thought to increase cell-to-cell contact, we further investigated the other cellular composition in the TME of the high Apical Junction Pathway score group, using xCell algorithm. The high Apical Junction Pathway score group was associated with significantly higher number of stromal cells, such as fibroblasts, adipocytes, endothelial cells including microvascular (mvE) and lymphatic endothelial (lyE) cells and pericytes, which are the cells contributing to angiogenesis and lymphangiogenesis (**Figure 3**). These findings suggested that Apical Junction Pathway is activated in gastric cancer with enhanced neovascularization and angiogenesis in the TME, leading to possible increased lymphatic as well as distant metastatic potential.

Epithelial mesenchymal transition as well as angiogenesis were activated in gastric cancer with high apical junction pathway score

Given that Apical Junction Pathway Score was associated with infiltration of stromal cells in-

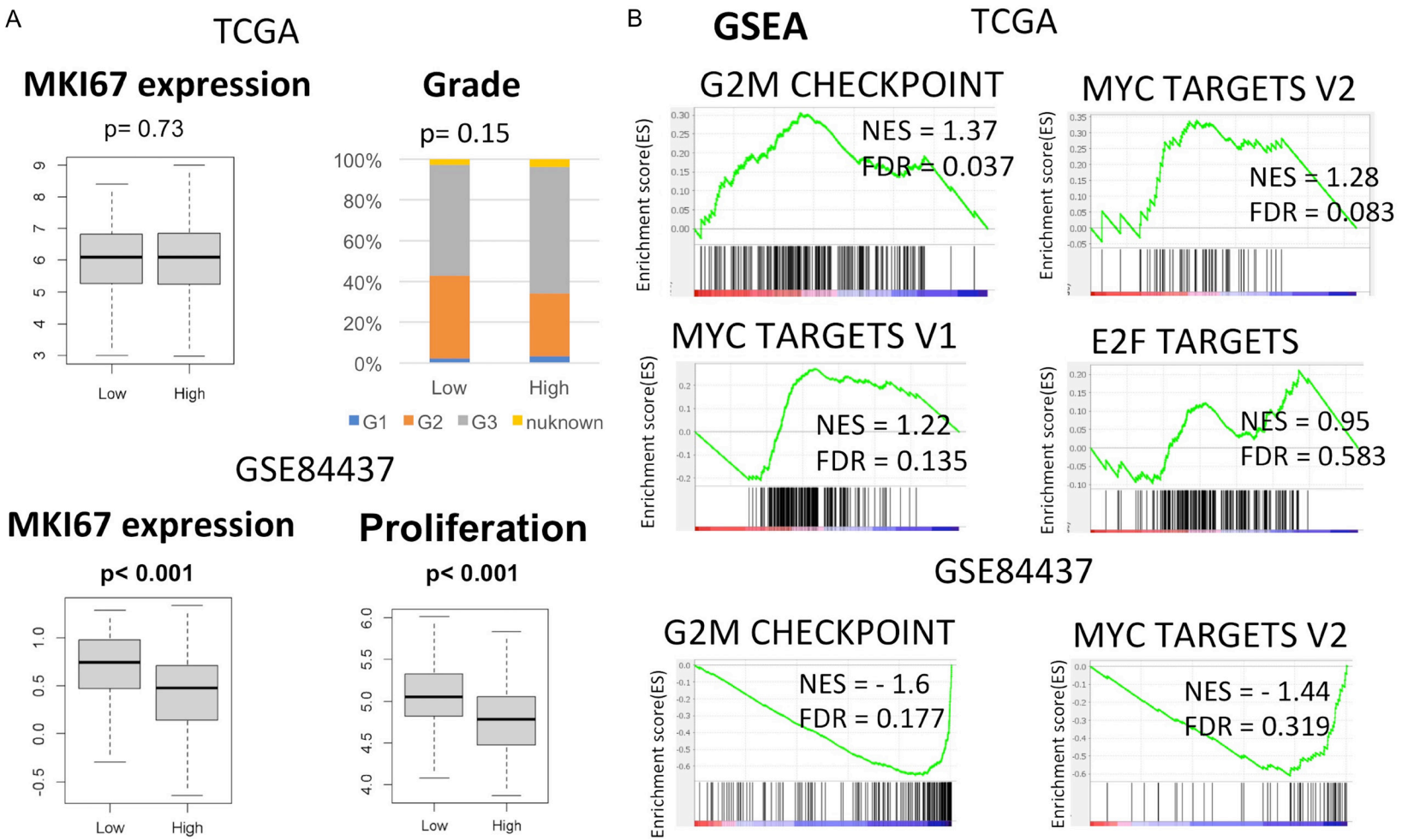

Figure 1. No strong association was observed between tumor proliferation and Apical Junction Pathway score. (A) With TCGA cohort, there was no difference in MKI67 expression and Grade between high and low Apical Junction Pathway score groups. With the GSE84437 cohort, while MKI67 expression and proliferation were higher in the low Apical Junction Pathway score group (P<0.001, respectively). (B) None of proliferation related gene sets, including G2M CHECKPOINT, MYC TARGETS V1/V2 and E2F TARGET were enriched in the high Apical Junction Pathway score group on GSEA (B). TCGA, the cancer genomic atlas; GSEA, gene set enrichment analysis.

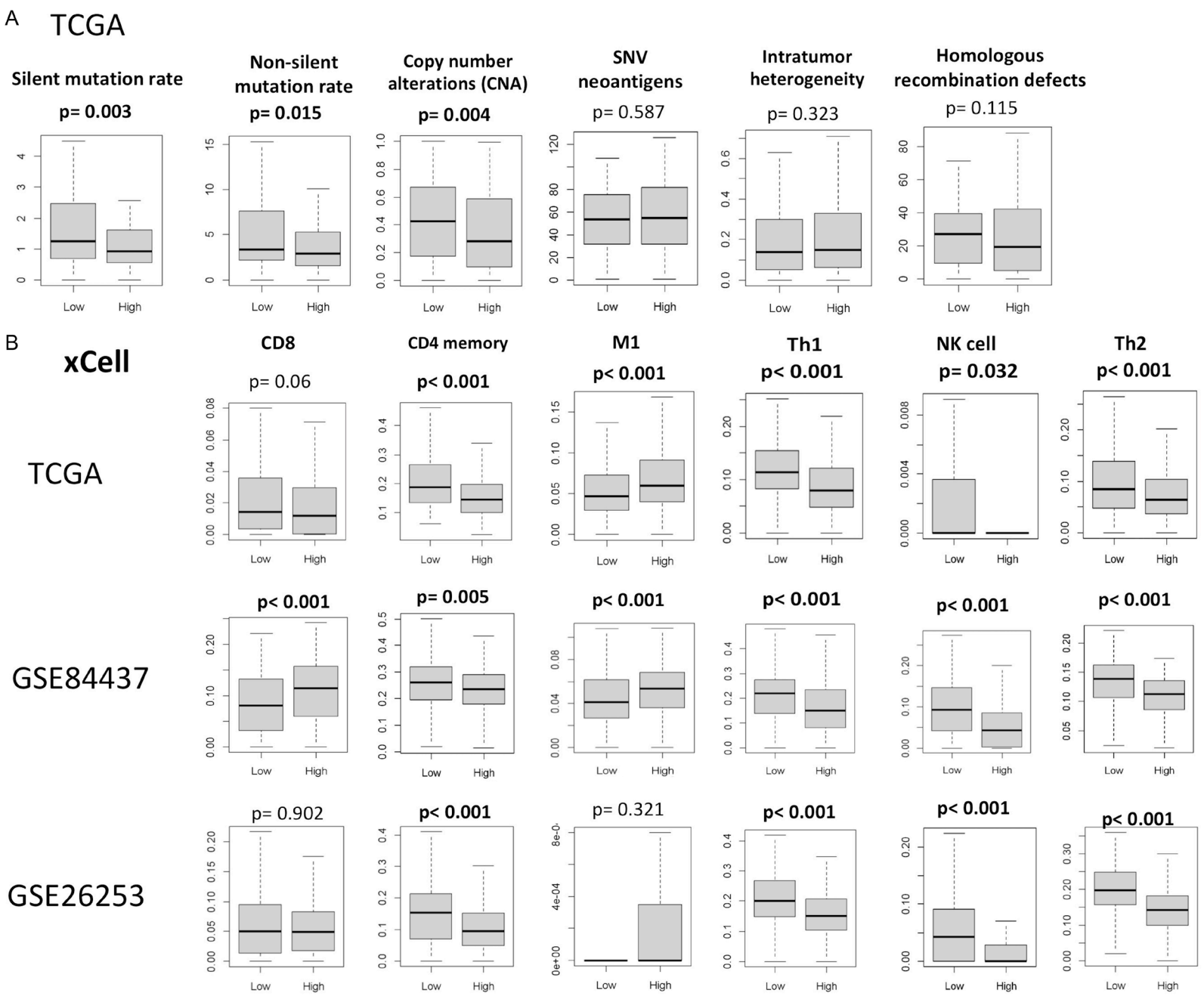
A
TCGA
Silent mutation rate
p= 0.003
Non-silent mutation rate
p= 0.015
Copy number alterations (CNA)
p= 0.004
SNV neoantigens
p= 0.587
Intratumor heterogeneity
p= 0.323
Homologous recombination defects
p= 0.115
Low
High
B
xCell
TCGA
GSE84437
GSE26253
CD8
p= 0.06
CD4 memory
p< 0.001
M1
p< 0.001
Th1
p< 0.001
NK cell
p= 0.032
Th2
p< 0.001
p< 0.001
p= 0.005
p< 0.001
p< 0.001
p< 0.001
p< 0.001
p= 0.902
p< 0.001
p= 0.321
p< 0.001
p< 0.001
p< 0.001

Figure 2. No strong association was observed between immunogenicity and Apical Junction Pathway score. A. With TCGA cohort, there were no consistent data to suggest strong immunity in the high Apical Junction Pathway score group. Silent and non-silent mutation rates as well as CNA are higher in the low Apical Junction Pathway score group, while neoantigens, intratumor heterogeneity, and homologous recombineation defects were not different between groups. B. xCell algorithm was used to exam immune cell composition in the TME of both high and low Apical Junction Pathway score groups. There were no consistent data in the immune cell composition based on the Apical Junction Pathway score; while some anti-cancer immune cells such as CD4 memory T cell, Helper T cell 1, and NK cells were high in the low score group in all three cohorts, CD8 T cells and M1 macrophages were rather higher in the high score group in only one cohort (GSE84437). TCGA, the cancer genome atlas; CNA, copy number alterations; TME, tumor microenvironment; NK, natural killer.

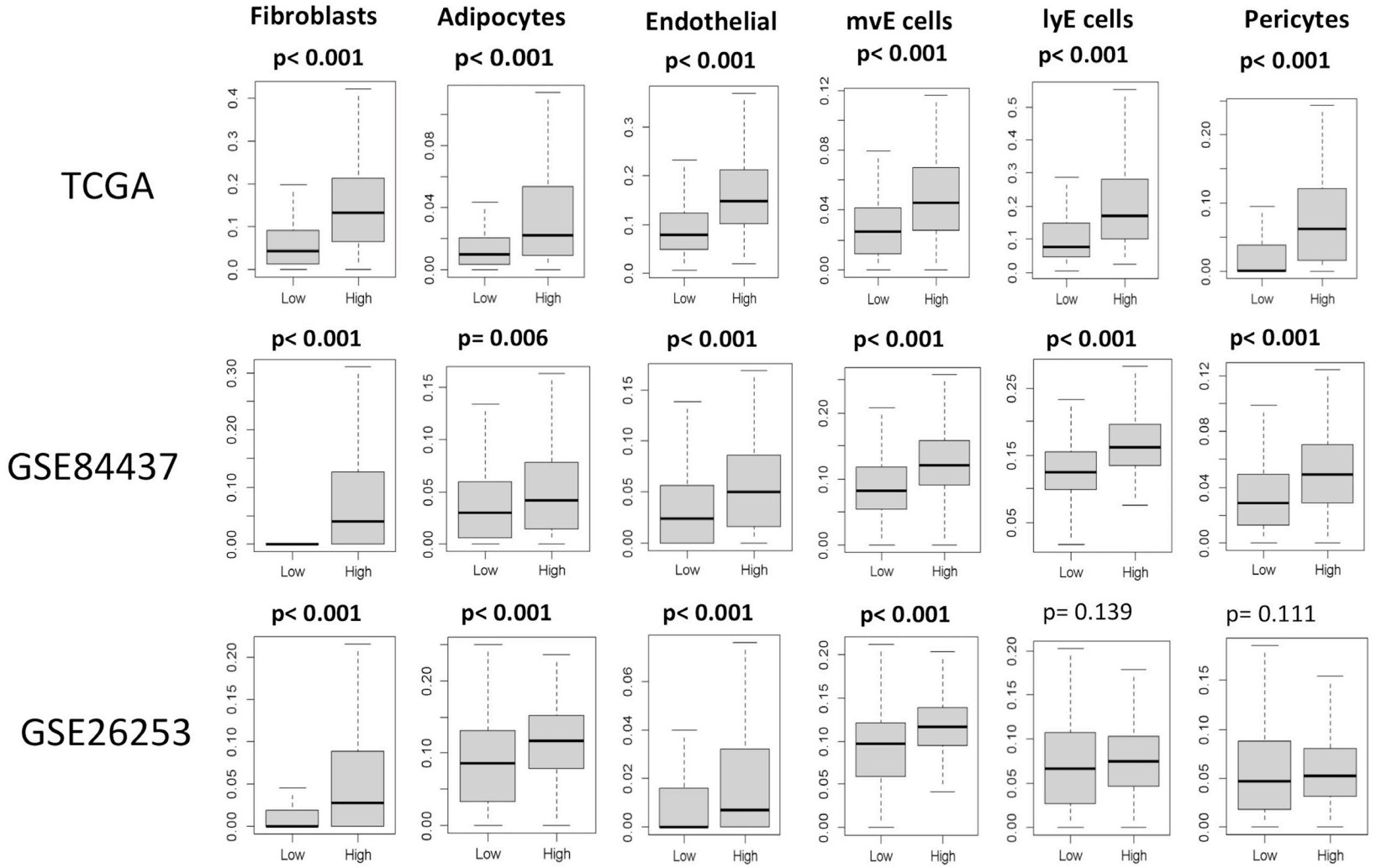

Figure 3. xCell algorithm demonstrated that gastric cancer with high Apical Junction Pathway score was associated with high fraction of stromal cells in TME, such as fibroblasts, adipocytes, endothelial cells and pericytes in all three cohorts. TME, tumor microenvironment.

cluding vascular endothelial cells and not with cell proliferation nor with immune cell infiltration, we utilized GSEA to investigate which pathways were enriched in the high Apical Junction score gastric cancer. The high Apical Junction Pathway score group significantly enriched EMT in addition to angiogenesis and myogenesis gene sets among the Hallmark collections consistently in both TCGA and GSE84437 cohorts (FDR <0.25; **Figure 4**). Hence, it is speculated that high Apical Junction Pathway score gastric cancers have higher metastatic potential due to EMT and angiogenesis.

Gastric cancer with high apical junction pathway score was associated with more aggressive clinicopathological characteristics as well as worse disease specific survival and overall survival

Given strong association between the Apical Junction Pathway score and cancer aggressiveness gene sets, we hypothesized that gastric cancer with high Apical Junction Pathway score would have aggressive clinical phenotypes. Indeed, gastric cancer with enhanced Apical Junction Pathway score was associated with higher American Joint Committee on Cancer (AJCC) T-category (P=0.003) and higher pathological stage (P=0.02) in the TCGA cohort. Similar results in AJCC T-category and N-category (P<0.01, respectively) were observed in the GSE84437 cohort (**Figure 5**). Furthermore, gastric cancer with high Apical Junction Pathway score demonstrated worse DSS and OS consistently in all three cohorts, TCGA, GSE84437 and GSE26253 (**Figure 6**). These results are likely because Apical Junction Pathway score was associated with aggressive biology and distant metastatic potential by angiogenesis and EMT.

Discussion

In the present study, we examined a total of 1,239 gastric cancer patients to investigate the association between Apical Junction Pathway score and gastric cancer biology as well as their prognosis, utilizing single-sample gene set expression scoring. Apical Junction Pathway score was defined as the GSVA score of the "HALLMARK_APICAL_JUNCTION" gene set using its median as a cut-off. Strength of GSVA is that it allows us to constellate multiple genes into one score instead of focusing on one specific gene, as the majority of signaling pathways are comprised of multiple gene sets. We found that gastric cancers with high Apical Junction Pathway score were not associated with enhanced cell proliferation or increased immune cell infiltrations in TME. On the other hand, the score was significantly associated with high infiltration of stromal cells in TME, such as microvascular and lymphatic endothelial cells as well as pericytes, which is likely reflection of increased angiogenesis and lymphangiogenesis. This result was echoed by GSEA results that demonstrated that the high score group was associated with increased metastatic potential with enrichment of EMT as well as angiogenesis gene sets consistently in two cohorts. These characteristics translated into more aggressive clinical features, such as advanced AJCC T stage, N stage, pathological stage, leading to worse disease specific and overall survival with high Apical Junction Pathway score.

Apical junction complex, integrated by the tight junction and the adherens junction, is a key component for intercellular connection and communication [2]. This system is crucial to maintain polarity of the cell by restricting the movement of lipids and proteins within the plasma membrane and passing ions and molecules through the paracellular pathway [41]. Furthermore, a diverse numbers of molecules of the apical junction complex, such as E-cadherin and claudins, are involved in the regulation of cell proliferation and gene transcription [42] and have active roles in cancer [3]. For example, E-cadherin is considered tumor suppressor and loss of E-cadherin expression is thought to trigger EMT [3, 43, 44]. Also, claudins are another intercellular adhesion proteins, which are expressed in tight junctions. Abnormal expressions of claudins have been reported in various cancer types and the function as tumor promoter or tumor suppressor depends on the claudin subtypes and cancer

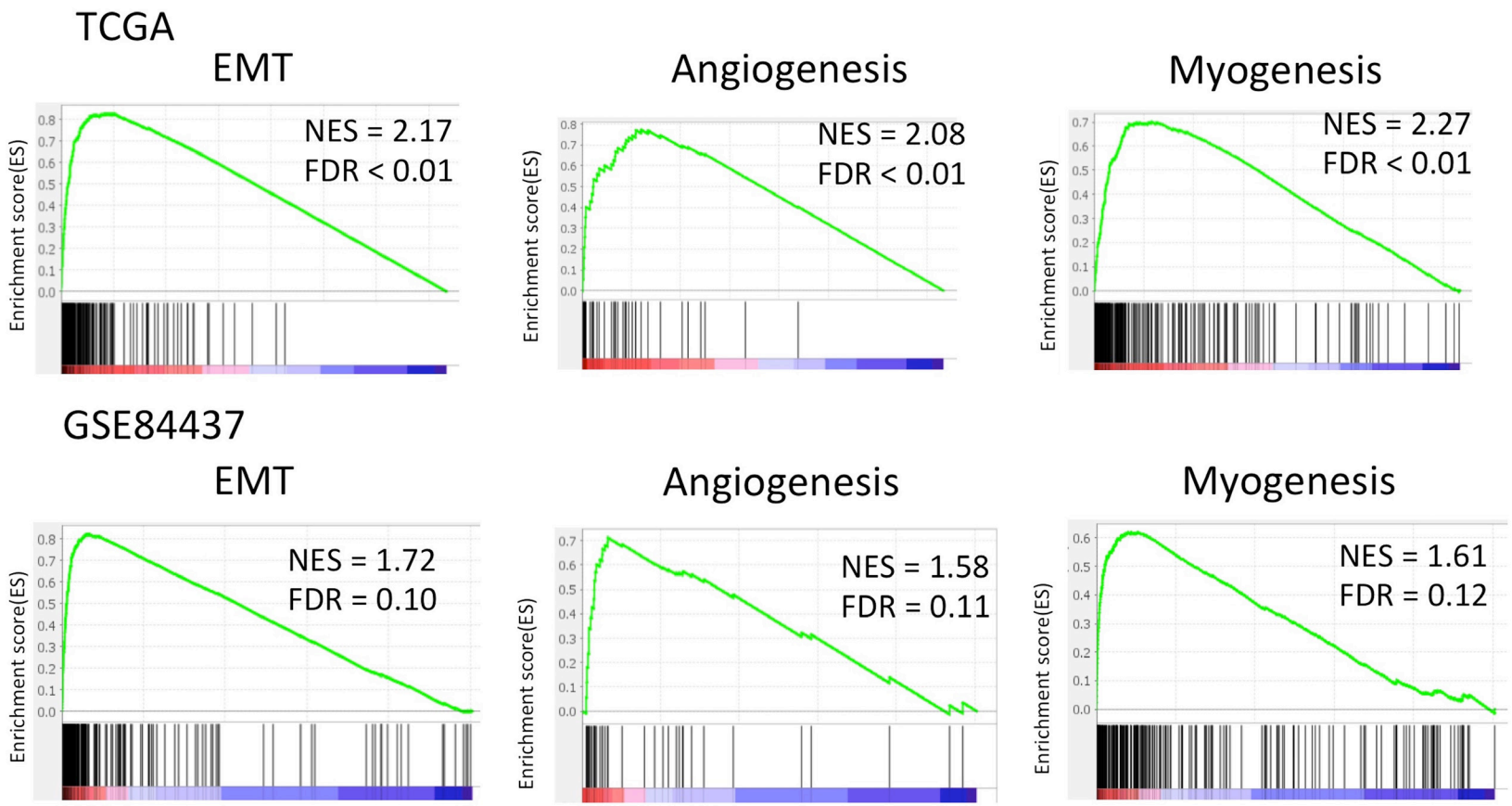

Figure 4. Expression of EMT, angiogenesis and myogenesis gene sets were enriched in high Apical Junction Pathway score gastric cancer on GSEA in both TCGA and GSE84437 cohorts (FDR <0.25, respectively). EMT, epithelial mesenchymal transition; GSEA, gene set enrichment analysis; TCGA, the cancer genome atlas; FDR, false discovery rate.

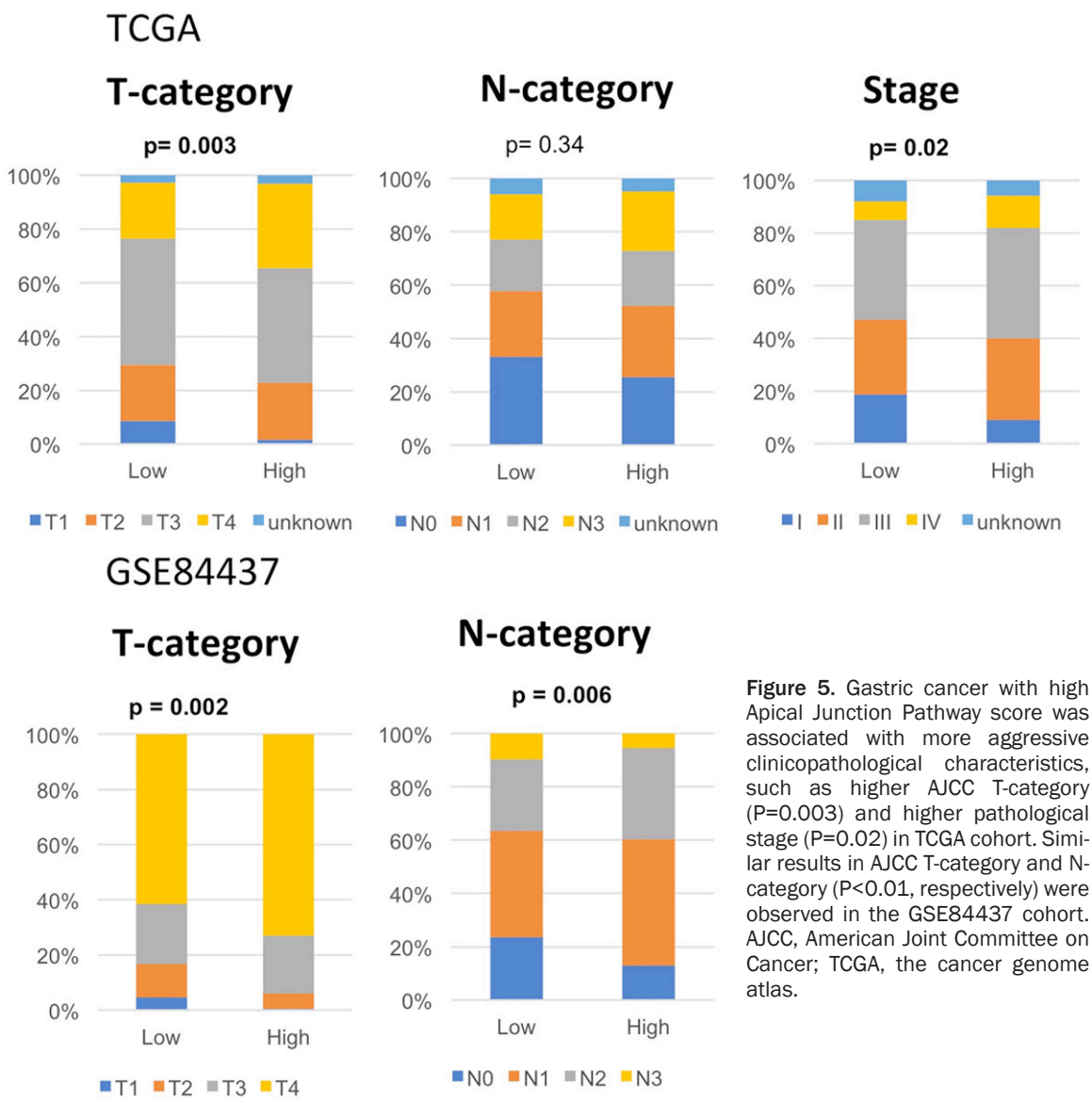

Figure 5. Gastric cancer with high Apical Junction Pathway score was associated with more aggressive clinicopathological characteristics, such as higher AJCC T-category (P=0.003) and higher pathological stage (P=0.02) in TCGA cohort. Similar results in AJCC T-category and N-category (P<0.01, respectively) were observed in the GSE84437 cohort. AJCC, American Joint Committee on Cancer; TCGA, the cancer genome atlas.

types [4, 45-47]. Herein, we examined correlation between E-cadherin (CDH1) and claudin (CLDN1) expression and Apical Junction Pathway score. While E-cadherin expression did not differ between the groups, claudin was expressed significantly more in the high Apical Junction Pathway score group (P=0.009; Figure S2). This result speculated that claudin might have been more associated with Apical Junction Pathway score, although it is difficult to conclude and many other genes could be contributing to the score given that GSVA score is taking multiple gene sets into account as a source.

Sustaining proliferative signaling or enhanced proliferation is one of hallmarks of cancer [48]. In general, the higher proliferation ability is, the more aggressive tumor biology becomes. Our group previously reported that the enhanced cellular proliferation pathways, such as G2M and E2F pathways, in breast cancers were associated with tumor aggressiveness and worse biology [7, 8]. Given possible association with apical junction pathway and cellular proliferation [3], we initially hypothesized that high Apical Junction Pathway score gastric cancer would have excessive intercellular connection and have increased proliferation ability, leading to worse outcome in this group. However, contrary to our initial hypothesis, Apical Junction Pathway score did not reflect enhanced cell proliferation ability or halt proliferation by

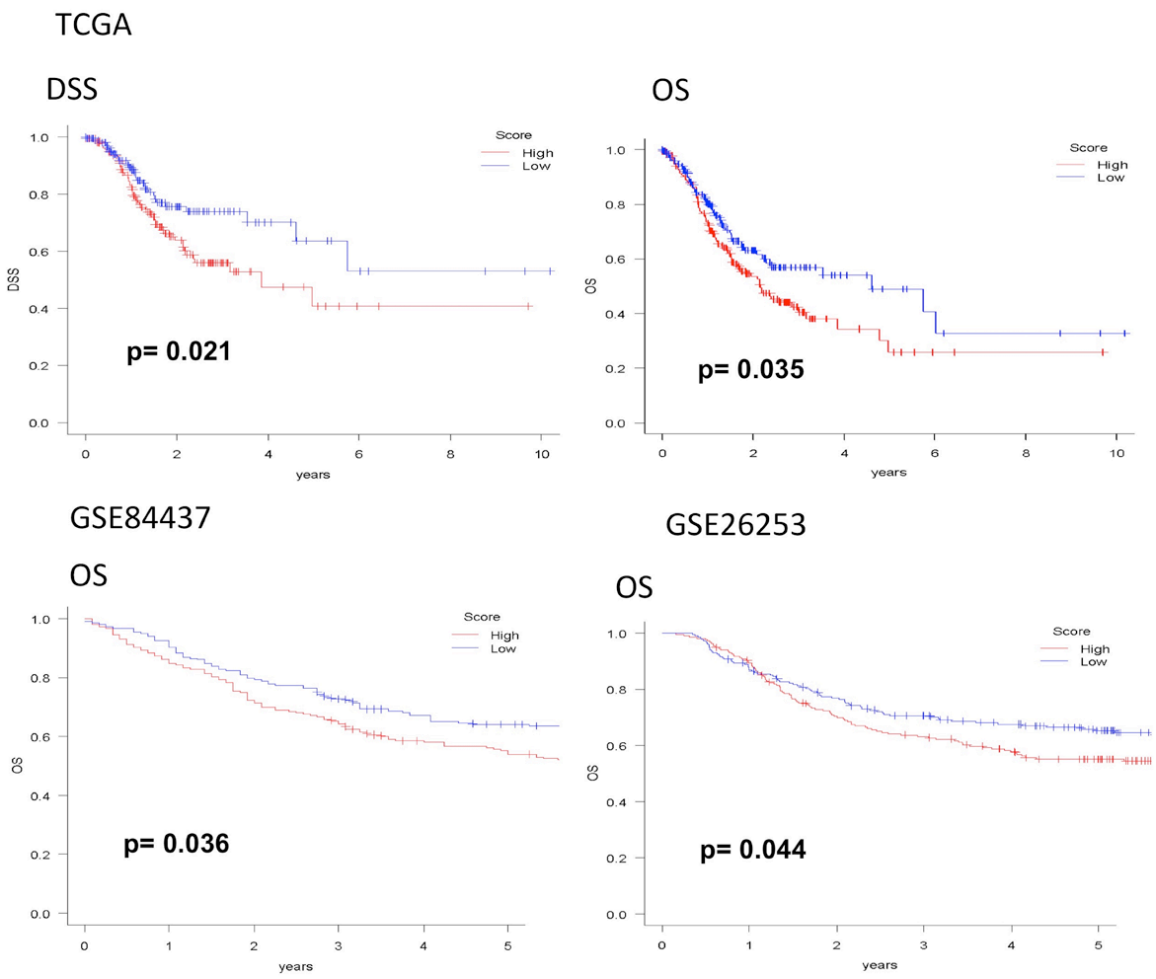

Figure 6. Gastric cancer with high Apical Junction Pathway score demonstrated worse DSS and OS in all three cohorts (P<0.05, respectively). DSS, disease-specific survival; OS, overall survival.

reaching confluency in gastric cancer. Since TME *In Vivo* is much more complexed than the cell dish *In Vitro*, a multitude of other factors beyond confluency are confounding the outcome of the Apical Junction Pathway score gastric cancer. In order to further investigate other possible factors, we examined the immune cell infiltration in the high Apical Junction Pathway score gastric cancer as immune cells are one of the major component of TME in multiple cancer types [24, 49, 50]. High Apical Junction Pathway score gastric cancer may have less favorable immune cells, such as CD4 memory T cells, NK cells and Helper T cell 1 (Th1), but we did not see any consistent data in xCell algorithm to draw strong conclusions; hence, Apical Junction Pathway score was not associated with uniform immune cell infiltration as well.

Instead, high Apical Junction Pathway score gastric cancer was associated with high infiltration of stromal cells, such as fibroblasts, adipocytes, endothelial cells, and pericytes, which suggested enhanced angiogenesis. This result was echoed by GSEA that enriched EMT as well as angiogenesis gene sets to high Apical Junction Pathway. These results indicated that the high Apical Junction Pathway score group would have increased metastatic potential through EMT as well as angiogenesis. EMT is a known key process of cancer invasion and distant metastasis [16, 51, 52]. EMT is the differentiation process through which epithelial cells gain mesenchymal phenotypes leading to invasion and dissemination, while normal epithelial cells are tightly bound to each other [51]. E-cadherin is an important component in the

EMT process as loss of E-cadherin expression is necessary to initiate EMT [43, 52]. On the other hand, cancer cells need to maintain epithelial phenotypes to colonize and develop distant metastasis [16]. As opposed to the binary condition, EMT is now considered as a spectrum of transition between the epithelial and mesenchymal phenotypes [51, 53, 54]. Additionally, angiogenesis is another well-known hallmark of cancer [48] and risk factors of cancer progression in various cancer types including gastric cancer [55, 56]. On the other hand, our group also previously reported that pancreatic cancer with mature vascularity is associated with better survival due to increased infiltration of anti-cancer immune cells [23]. Hence, angiogenesis could be context dependent. The present study demonstrated that angiogenesis was likely the cause of poor survival in the high Apical Junction Pathway score group.

Since the Lauren classification [57] or any other gastric cancer pathological classification [58] information was not available in the present study, we were unable to calculate Apical Junction Pathway score on diffuse type gastric cancer, also known as linitis plastica. One of main clinical characteristics of diffuse gastric cancer is fibrotic transformation of the stomach with submucosal tumor infiltration, with significantly worse prognosis than the intestinal type [59]. With the results from our study, we cannot help but speculate that Apical Junction Pathway score would be extremely elevated in diffuse type gastric cancer. Furthermore, with apical junction complex being a main component of intercellular connection and drug permissibility, we plan to further investigate association between Apical Junction Pathway score and chemotherapy response, as there are currently no biomarkers available to guide chemotherapy regimens.

There are a few limitations in the present study. While it is extremely useful to have clinicopathological with survival data attached with transcriptomic information such as TCGA, the publicly available databases have their own limitations, such as short term follow up, or limited number of patients. Also, this study was based on gene expression of the solely surgically resected primary tumor on all cohorts; thus, the Apical Junction Pathway score might not be applicable to metastatic disease sites. Similarly, even though we utilized three different cohorts, due to retrospective nature, selection bias is possibly present as well. Further, the present study does not include any In Vitro or In Vivo experiments; therefore, all our findings are based on exclusively association. Hence, it could possible that enhanced Apical Junction Pathway score may reflect a simple biomarker of advanced gastric cancer, instead of causation of increased metastatic potential. In order to further investigate the clinical relevance of Apical Junction Pathway score, the experimental approach with clinical correlation will be required.

Conclusions

In the presented study, enhanced Apical Junction Pathway score gastric cancer was found to have worse survival than the low score group. This was likely due to increased metastatic potential through EMT and angiogenesis instead of increased cell proliferation. In conclusion, Apical Junction Pathway score could be considered for a prognostic biomarker for gastric cancer.

Acknowledgements

This work was supported by National Institutes of Health (NIH) grant R01CA251545, R01CA250412, R37CA248018, R01EB0295-96, and Department of Defense Breast Cancer Research Program grant W81XWH-19-1-0111 and W81XWH-19-1-0674 to K.T. and National Cancer Institute (NCI) grant P30CA016056 involving the use of Roswell Park Comprehensive Cancer Center's Shared Resources.

Disclosure of conflict of interest

None.

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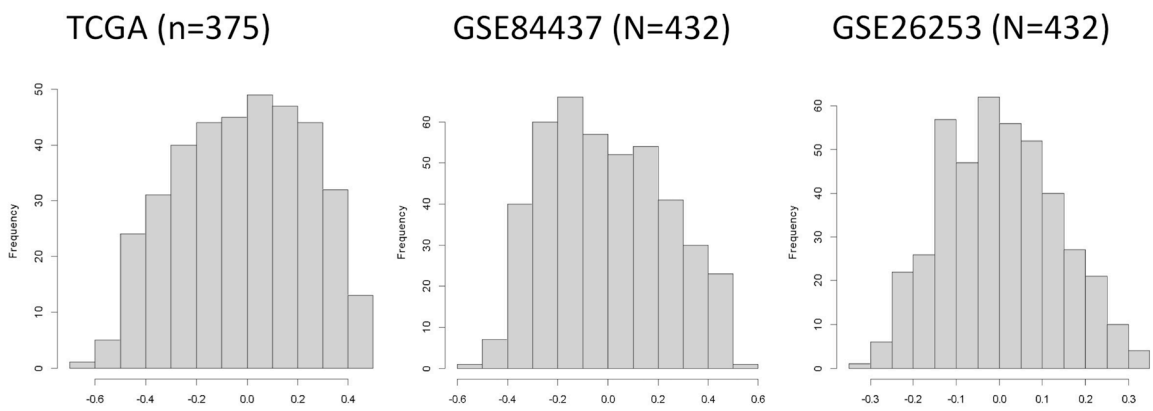

Figure S1. Apical Junction Pathway score was calculated in the patients with gastric cancer in all three cohorts using the GSVA score based on the Hallmark Apical Junction gene set. Shown is a histogram on the each cohort. *GSVA,* gene set variant analysis.

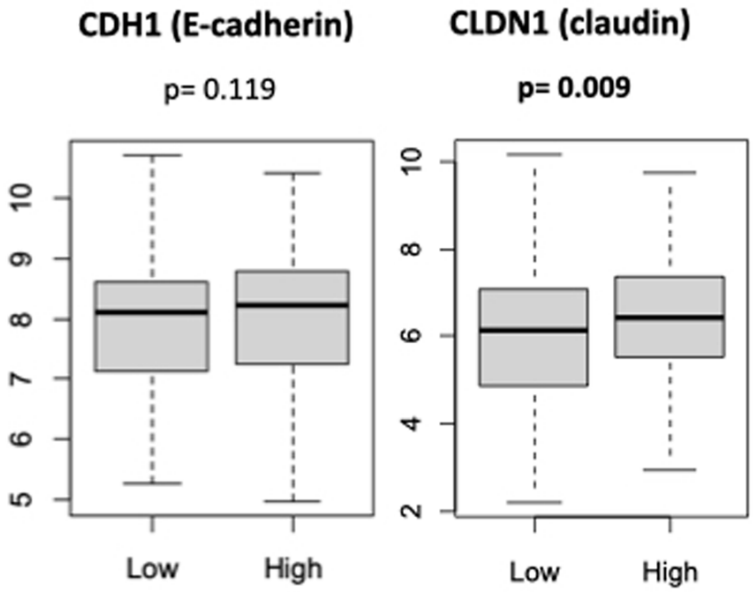

Figure S2. E-cadherin (CDH1) and Claudin (CLDN1) expression were compared between the low and high Apical Junction Pathway score groups using the TCGA cohort. While CDH1 was not different between the groups, CLDN1 expression was significantly higher in the high Apical Junction Pathway score (P=0.009). TCGA, the cancer genome atlas.